HETEROKARYOSIS IN STREPTOMYCES

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The actinomycetes are a group of bacteria (Bergey's Manual, 6th edition) whose mycelial habit resembles that of the molds. This growth pattern lends itself to *heterokaryosis*, the genetic cooperation of diverse nuclei in a cytoplasmic field. The importance of heterokaryosis in evolutionary and laboratory experiments has been amply stressed by other workers (Beadle and Coonradt, 1944; Pontecorvo, 1946.)

MATERIALS AND METHODS

Processes of genetic recombination can be revealed by the occurrence of new types in mixed cultures of strains with different genetic markers. Growth-factor-dependent or auxotrophic mutations are convenient markers, for the wild type, or prototrophic, combination can be selectively detected on minimal medium (Lederberg *et al.*, 1951).

Strains of Streptomyces were isolated from soil samples collected at Evanston, Illinois, and Madison, Wisconsin. The isolates were chosen for their ability to grow rapidly and sporulate well on a glucose-salts medium at 30 to 37 C. The differential characteristics of the initial stocks and their mutant derivatives are given in table 1.

Cultures were maintained on glucose-salts medium of the following composition: glucose, 2g; KNO_3, 0.2g; K_2HPO_4, 0.2g; $MgSO_4 \cdot 7H_2O$, 0.05g; $Ca(OH)_2$, 0.002g; with or without agar (Difco), 2g; distilled water, 100 ml. For complete medium 0.1 per cent yeast extract was added to the minimal substrate. Occasionally, nystatin ("mycostatin," E. R. Squibb and Sons, New York) was added to agar media at a concentration of 10 units/ml to effectively suppress mold contamination.

With the usual precautions of aseptic technique, spores were scraped from agar plates and suspended in water. The spores were dispersed with a tissue homogenizer or by trituration with a pipette. Large particles were removed by filtration through cotton. With this method, single spores account for at least 90 per cent of the plating units. The remainder are aggregates of 2 to 3 spores and minute hyphal fragments. Plates were streaked or else a measured volume of the spore suspension was spread with a bent glass rod. They were incubated at 30 C until ready for harvest.

Auxotrophic mutants were obtained after treatment with ultraviolet light. Seeded complete agar plates were exposed to a Hanovia high pressure mercury lamp (Alpine Burner Type SH, 125 watts; for 20 sec; at 16 cm) which left 0.1 per cent surviving colony-formers. To detect auxotrophic mutant colonies or sectors, the surviving colonies on complete agar were allowed to sporulate, and then replica plated (Lederberg and Lederberg, 1952) onto minimal agar (figure 1). Each presumptive mutant was replated and retested several times. About 0.1 per cent of the ultraviolet survivors were stable mutants, including auxotrophs of the following growth factors: arginine, methionine, tryptophan, biotin, riboflavin, uracil, leucine, isoleucine, guanine and uracil, isoleucine and valine, glutamate or aspartate or proline, serine or cystine, adenine or guanine, and purine (adenine or guanine or hypoxanthine or xanthine). Growth-factor supplements were added when indicated at a concentration of 10 mg/L.

In addition, stable morphological variations in the soluble pigment, color of the aerial mycelium, and colonial character occurred, but have been de-emphasized because recombinant phenotypes cannot be predicted or readily selected.

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TABLE 1

Principal stocks and their ancestors

Designation	Source	Genotypic Formula†
WAc-34	*Streptomyces griseus** fresh isolate (Evanston, Ill.)	T $M^sB^sS^sV^r$
WAc-74	WAc-34	$Arg^-Pu_1^-T\ M^fB^{r1}S^sV^r$
WAc-75	WAc-74	$Arg^-Pu_1^-T\ M^fB^{r1}S^rV^r$
WAc-40	WAc-34	$Try^-T\ M^sB^sS^sV^r$
WAc-59	WAc-40	$Try^-Pu_2^-T\ M^sB^{r1}S^sV^r$
WAc-77	WAc-59	$Try^-Pu_2^-T\ M^sB^{r1}S^rV^r$
WAc-41	WAc-34	$Asp^-T\ M^sB^sS^sV^r$
WAc-86	*Streptomyces griseus**, fresh isolate (Madison, Wis.)	T $M^sB^{r5}S^sV^s$
WAc-32	*Streptomyces cyaneus** fresh isolate (Evanston, Ill.)	G $B^sS^sV^r$
WAc-45	WAc-32	$Leu^-G\ B^sS^sV^r$
WAc-52	WAc-45	$Leu^-G\ B^sS^rV^r$

* According to Waksman and Lechevalier (1953).

† The abbreviations employed are:

T (tan aerial mycelium);

G (green aerial mycelium);

M^s (sharp colonial margin);

M^f (fuzzy colonial margin);

B^s (bacitracin sensitive);

B^{r1} and B^{r5} (resistant to 1 and 5 units/ml bacitracin, respectively);

S^s and S^r (streptomycin sensitivity and resistance);

V^r and V^s (resistance and sensitivity to actinophage V104-86);

Arg^- (requires added arginine);

Try^- (requires added tryptophan);

Leu^- (requires added leucine);

Pu_1^- and Pu_2^- (independent mutations each satisfied by either adenine, guanine, xanthine or hypoxanthine);

Asp^- (requires aspartic acid, or glutamic, or proline).

Drug resistant markers were selected as spontaneous mutants, at 200 mg/L dihydrostreptomycin sulfate or 1 and 5 units/ml bacitracin. Streptomycin-resistant strains grow more slowly than their sensitive counterparts, and on continued transfer in the absence of the drug may revert to sensitivity. Bacitracin-resistant lines grow as well as the sensitive lines and show no tendency to revert.

RESULTS

Syntrophism. Many combinations of auxotrophs show syntrophism, i. e., cross-feeding through the medium. Mixed platings of spores from these auxotrophs characteristically grow diffusely on minimal medium after 4 to 15 days incubation. Converging streaks of synergizing pairs grow progressively from the point of nearest approach (figure 2). Syntrophic cooperation has been observed over distances of not less than 5 cm. Syntrophism complicates the detection of more intimate associations, but can be minimized by an empirical choice of strains.

Heterokaryosis. Several mutants which exhibit little or no syntrophism are particularly suited to the study of nuclear interaction. They include WAc-74, WAc-77, and WAc-45 (table 1).

Spore mixtures of WAc-74 ($Arg^-Pu_1^-S^sM^f$) and WAc-77 ($Try^-Pu_2^-S^rM^s$) gave prototrophic colonies (figure 3) on minimal medium after 8 to 15 days, compared to 3 days for the ancestral wild type. The highest relative yield of prototrophs was 1 per 1000 spores plated. The proportion of parental spores and population density affected the yield, which was largest at 1 WAc-74:3 WAc-77, with a total of 10^4 spores per plate. The relative yield declined with fewer or more spores. In very populous plates (5×10^7 spores/plate) a diffuse confluent growth developed, probably due to syntrophism. These auxotrophs grow slightly on minimal substrate (WAc-74 more than WAc-77), therefore quantitative inferences based on the number of spores plated, rather than the later total growth, are unreliable.

Less than 0.1 per cent of the spores from prototrophic colonies developed on minimal medium. Iterated replatings, through as many as seven cycles, always gave essentially the same result. The prototrophs that were obtained may be attributed to residual mycelial fragments rather than spores. The prototrophic colonies from these platings developed more slowly than the ancestral wild type, but generally more rapidly (and more frequently) than new interactions of freshly mixed auxotrophic spores. Altogether, spores from 1384 prototrophic colonies have been replated; none proved to be stable, i. e., recombinants rather than heterokaryons.

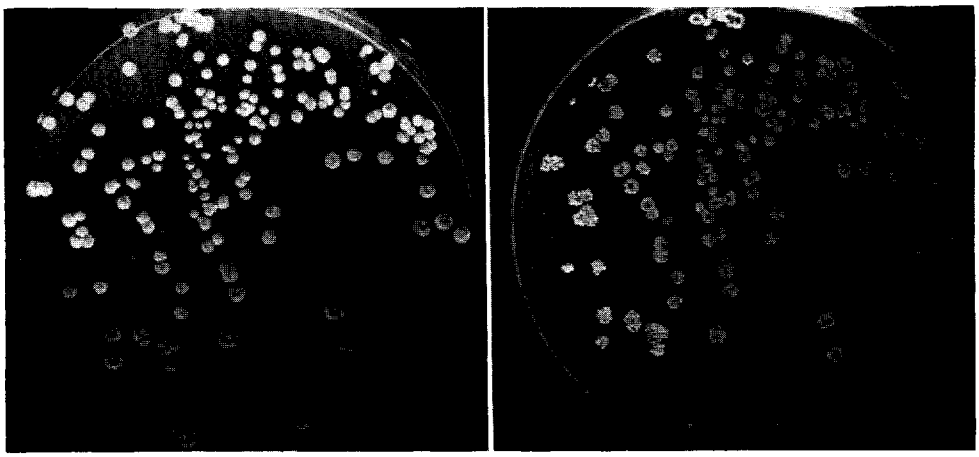

Figure 1. Replica plates of *Streptomyces griseus*, WAc-86. *A*. Original plate on complete agar, showing sporulating colonies. *B*. Replica plate to minimal agar. All the colonies are prototrophic, but some near the edge were not transferred by the velvet.

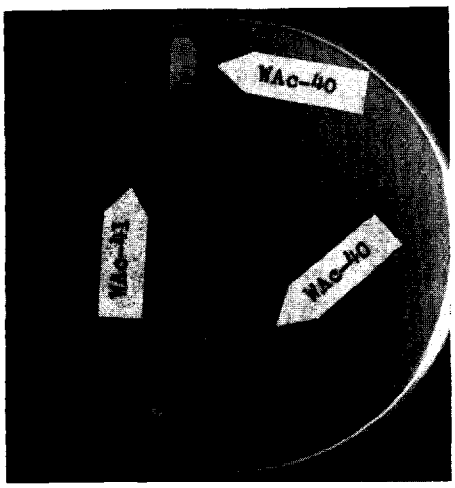

Figure 2. Syntrophism of WAc-40 and WAc-41. Spore suspensions were streaked on minimal agar as indicated. The plate was incubated for nine days.

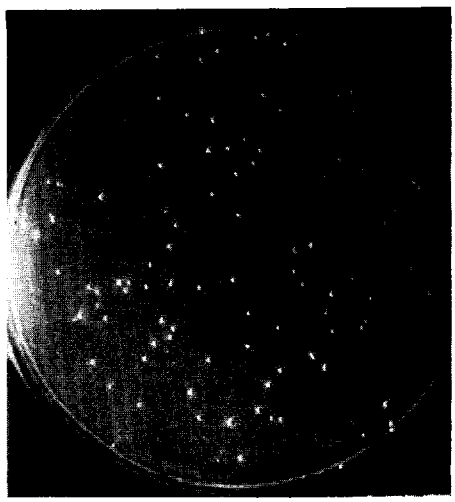

Figure 3. Discrete prototrophic colonies from a mixed plating of WAc-74 and WAc-77 on minimal agar. The parental auxotrophs are responsible for the background growth.

Whereas the prototrophic quality was rarely if ever transmitted through spores, it could often be propagated by pieces of vegetative mycelium. Pieces transferred to minimal medium + streptomycin failed to grow, suggesting that as in *Escherichia coli* (Lederberg, 1951), streptomycin-resistance is recessive to sensitivity.

Similar experiments, in which the streptomycin marker was reversed, were conducted with WAc-59 and WAc-75. Again only the parental types were recovered.

Conditions affecting prototroph formation were examined. Mixtures of minimal and complete agar (0:20, 1:19, 2:18, . . . , 18:2, 19:1, 20:0) were used as the test substrates. Progressively older spore-crops were plated daily on minimal agar over a period of 3 to 11 days. No stable prototrophic colonies have been obtained by this procedure. Complementary mixtures were incubated at 15, 20, 25, 30, and 37 C on minimal plates. No prototrophs developed

at 37 C even though both parents and their prototrophic ancestors grow well at this temperature on appropriate media. Prototroph formation at temperatures of 25 C or below was progressively delayed but occurred in the same yield. Addition of NaCl, KCl, $MgSO_4$, and $CaCO_3$ up to 0.1 per cent had no effect. Prototroph formation was delayed in semisolid agar (0.6 per cent) and occurred infrequently in broth cultures, whether stationary, rolled, bubbled, or shaken. Complementary mycelia grown separately in complete broth, then centrifuged together prior to plating on minimal substrate did not yield as many prototrophs as the same combination did when spores were plated directly to minimal agar.

Thus WAc-74 and WAc-77 when grown in intimate association on minimal agar produce discrete colonies (figure 3). These prototrophic mycelia may be tentatively considered as heterokaryons. Spores from these colonies engender only the parental types, hence are homokaryotic and presumably uninucleate or derived from a single nucleus.

Isolation of single hyphae from heterokaryons. Heterokaryosis has been proved by demonstrating both unaltered parental genotypes within a single hyphal segment. The following procedure was used with the exceptions discussed later. Prototrophic colonies from WAc-74 and WAc-77 on minimal agar were flooded and scraped to wash away as much aerial growth as possible. The underlying vegetative mycelium was dug out of the agar and minced with a platinum spatula to make a suspension of hyphal fragments. Small drops were placed on an oil-coated cover glass which was then inverted over an oil chamber for micromanipulation (de Fonbrune, 1949). The manipulations were conducted with hand-drawn micropipettes under dark field microscopy at 150×. Single hyphal bits, from 3 to 15 μ in length, either simple or branched, were teased off and transferred to individual drops of complete broth. Each isolate was checked at 600× and 1500× under dark phase contrast. About three-fourths of the fragments were viable; the smaller fragments, which were usually handled more strenuously, tended to be less viable. Where a microcolony developed from a reliable isolate, it was invariably single and symmetrical, corroborating its origin from a single hyphal fragment. As a further check on the technique, in some runs, spores of WAc-32 were purposely added to suspensions of the heterokaryons. This contaminant was never recovered from the single-hyphal isolations.

The microcolonies reached a diameter of 1 to 2 mm after 2 to 3 days and were then transferred manually to complete agar plates where they were allowed to develop to maturity. The spores from these primary colonies were thinly seeded onto minimal and complete agar. On minimal agar, the spores gave no prototrophs, which is consistent with the results of previous platings of spores from heterokaryons. The secondary colonies which developed on complete agar were replica plated to differentiating media and proved to correspond to one or the other parental type in regard to nutrition, streptomycin response, and colonial morphology. Direct platings to differentiating media, and replica platings therefrom, likewise revealed only parental types. As shown in table 2, 97 viable hyphae yielded 36 homokaryons, i. e., primary colonies which gave only one or the other parental genotype. The remaining 61 hyphae were heterokaryons, i. e., yielded both parental types. About 20 secondary colonies were replica plated in testing each primary colony. The ratio of parental components averaged about 2 WAc-77 for 1 WAc-74.

In two of the runs the isolates were made to minimal broth and subsequently transferred to minimal agar, instead of to the usual complete media (table 2). Most of the fragments developed (13 of 15) in the minimal substrates and were therefore prototrophic. All of these were verified to be heterokaryons by the criteria described above.

In another two runs, the sources of the isolated fragments were microcolonies that had developed from single hyphal bits in complete broth. Since 3 of 7 isolated hyphae were still heterokaryotic, this association is relatively stable in vegetative growth in contrast to regular dissociation in the spores. However, sectored colonies from which the parental types could be re-isolated occurred frequently in the course of vegetative growth, both on complete and minimal agar.

To detect recombinant nuclei in the primary colonies, the spores were plated directly to differentiating media. No stable new types were identified although "anomalous heterokaryons" were isolated (their incidence is shown in table 2). The anomalies occurred individually among a few hundred tested secondary colonies. One of

TABLE 2

Hyphal fragments isolated from prototrophic colonies

Combination		Fragments		Heterokaryons	Homokaryons	
Parent A	Parent B	Isolated	Viable		A	B
$Try^-Pu_2^-S^rArg^+Pu_1^+$	$Try^+Pu_2^+S^sArg^-Pu_1^-$	17	13	11	2	0
		15	11	10*	1	0
		13	7	4*	2	1
		12	10	6	2	2
		9	5	0	4	1
		8	8	7	0	1
		8	4	1	3	0
		7	6	3	2	1
		6	6	0	6	0
		5	3	1	1	1
		4	4	2	0	2
		8†	8	8*	0	0
		7†	5	5*	0	0
		5‡	3	2	0	1
		4‡	4	1	3	0
	Total	128	97	61	26	10
$Try^-Pu_2^-S^rLeu^+$	$Try^+Pu_2^+S^sLeu^-$	8	8	8	0	0
		10	7	7	0	0
		5	5	3	0	2
		7	5	5	0	0
		3	2	0	1	1
	Total	33	27	23	1	3
$Arg^-Pu_1^-Leu^+$	$Arg^+Pu_1^-Leu^-$	18	16	7	8	1

Fragments from the vegetative mycelia of prototrophic colonies were isolated by micromanipulation and grown to maturity. The colonies that developed were analyzed to determine if the original fragments were homo- or heterokaryotic.

* This figure includes one (or two) "anomalous heterokaryons" found among several secondary colonies examined.

† These fragments were isolated and grown on minimal rather than complete substrate.

‡ These fragments were isolated into complete broth from microcolonies which had developed in complete broth from a single hyphal fragment.

these grew on the doubly supplemented media that suffice for each parent (ArgPu or TryPu) but would not grow on any single supplement with or without other growth factors. This could not be a simple reversion.

A second type of "anomalous heterokaryon" would not grow on either the single or double supplements, but would grow on the triple supplement (ArgTryPu). Combinations with either parental line on minimal plates gave prototrophs; hence this cannot be construed as a simple recombinant.

No definite interpretation for these anomalies can be offered at present. Although the anomalous phenotypes were regularly transmitted through spores, cultures maintained on complete medium eventually sectored into the parental types.

Heterokaryosis in other strains. The strains employed in the preceding experiments were derived from a common ancestor. Conceivably, streptomycetes are heterothallic and/or contain incompatibility factors. Therefore, combinations of Wac-45 + WAc-74 and WAc-45 + WAc-77 were tested with substantially the same results as previously described for WAc-74 + WAc-77. Prototrophs of intermediate color were formed on minimal medium by both of these combinations. Spores from the prototrophic colonies yielded only parental types. Isolation of single hyphal bits demonstrated the intimate association of the parental genotypes (table 2).

Survey to detect gene recombination. Although there was no indication of synkaryotic recombination in the previous results, several strains were screened against genetically marked tester

strains. The selective markers which were used included streptomycin, bacitracin and phage resistance and prototrophy.

In one series of tests an auxotrophic, streptomycin-resistant stock was plated with a prototrophic streptomycin-sensitive strain. The recombinant type selected for was a streptomycin-resistant prototroph. Fifteen strains isolated from the soil were tested against WAc-77, WAc-75 and WAc-52. Techniques described in a previous section were applied: (1) cultures were grown separately in broth, then centrifuged together; (2) cultures were grown together in broth; (3) cultures were grown together on complete plates and the spores tested; and (4) mixtures were made directly on the selective substrate, which was minimal-streptomycin agar. No recombinant types were found.

In a second series, auxotrophic, phage-resistant Streptomyces were mixed with a prototrophic phage-sensitive tester (WAc-86). The previously described cultural methods were used. The selective plating consisted of minimal substrate plus actinophage V104-86, isolated from soil, and maintained on WAc-86. The combination of the test organism (WAc-86) with mutant strains of 10 different lines yielded no recombinants.

In a third series, phage-resistant, bacitracin-sensitive strains were mixed with a phage-sensitive, bacitracin-resistant tester (WAc-86) and plated after various procedures on the selective medium which contained bacitracin + phage. No recombinants were found among the 12 combinations analyzed.

DISCUSSION

Heterokaryosis provides a means of genetic interaction whereby diverse nuclei can, by cooperation, accomplish ends neither could alone. Heterokaryosis has the adaptive value of maintaining alternative genes which can be called into action. This intimate association sometimes leads to cryptic sexuality or "parasexual" recombination (Pontecorvo, 1954).

In the absence of other genetic interactions, heterokaryosis can be exploited in physiological and hereditary analyses. For example, non-identity of similar but independent mutations is indicated by the formation of effective heterokaryons. Syntrophism furnishes a similar criterion. Neither of these methods discloses whether physiological difference is based on genetic non-allelism.

The hyphae of Streptomyces are often closely appressed if not actually fused (Carvajal, 1946; Jones, 1950). However cytological proof of true fusion is difficult because of the minute size of the vegetative hyphae. Hyphal fusion has been confirmed by demonstrating that genetically different nuclei can be isolated from a single hyphal fragment of a prototrophic colony, which developed in a mixture of complementary auxotrophs. Even though occasional septae do occur, at least in older areas, the mycelium of Streptomyces is, for the most part, coenocytic (Kleineberger-Nobel, 1947). Therefore, the hyphal fragments that were isolated by micromanipulation are considered to be single "cells."

Genetically marked strains of Streptomyces have been brought together under different experimental conditions. Complementary auxotrophs form heterokaryons which dissociate into the parental components during sporulation. Within the heterokaryon nutritional independence is dominant to dependence and streptomycin sensitivity is dominant to resistance.

Certain "anomalous heterokaryons" have been found. They can not subsequently develop on minimal medium although originally isolated from that substrate. Both intact parental genomes seem to be present and transmitted through the spores of these anomalous isolates, but do not interact effectively. Further study is required to verify whether these are truly heterokaryons, or dikaryons, heterozygotes or some less evident mode of genetic association.

No stable recombinants have been recovered from the heterokaryons or from mixtures specifically designed to detect recombinational mechanisms. The survey techniques involved streptomycin-prototroph selection, as well as actinophage-prototroph and actinophage-bacitracin selections.

Sermonti and Spada-Sermonti (1955) have recently reported genic recombination in *Streptomyces coelicolor*, by methods essentially similar to those reported here. Although no details were given as to the time and site of syngamy and meiosis, reduction did occur prior to spore-formation. If their results can be correlated with ours, heterokaryosis would be a plausible preliminary to karyogamy. Whether this occurs in the vegetative or aerial mycelium, or whether

recombination has some other basis, is not yet indicated.

The reason for the difference in recombinational patterns in *S. coelicolor* versus *Streptomyces griseus* and *Streptomyces cyaneus* is not known.

SUMMARY

Heterokaryosis in the Streptomyces has been established by analyzing prototrophic colonies formed from intimately associated combinations of complementary auxotrophs on minimal agar. Spores from prototrophic colonies were unable to propagate that character whereas mycelial fragments often could. Genetically, the spores corresponded to one or the other parental type, hence are homokaryotic. The heterokaryotic nature of the vegetative mycelium was verified by demonstrating the presence of both intact parental genomes in a single hyphal fragment isolated by micromanipulation. Infrequent "anomalous heterokaryons" were found that could not synergize effectively even though both parental genomes were present. No stable recombinants were detected in these experiments or in survey experiments designed especially for that purpose.

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